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Changes in flavonoids secreted by *Phaseolus vulgaris* roots in the presence of salt and the plant growth-promoting rhizobacterium *Chryseobacterium balustinum*

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ABSTRACT

Root exudates affect the concentration of nutrients in the plant rhizosphere and indirectly enhance microbial activity. Flavonoids are polyphenolic compounds that play an important role as signal molecules in the early stages of the symbiosis between legumes and rhizobia. Flavonoids from root exudates of *Phaseolus vulgaris* cv. Bush Blue Lake obtained under control conditions, salt stress (50 mM), and/or the presence of the plant growth-promoting rhizobacterium (PGPR) *Chryseobacterium balustinum* Aur9 were analyzed by HPLC coupled to ESI-MS/MS. Six flavonoids were identified in the root exudates of *P. vulgaris* cv. BBL: naringenin, isoliquiritigenin, quercetin umbelliferone, 7′,4-dihydroxyflavone, and hesperetin. The latter three flavonoids have not previously been reported in bean root exudates. The presence of *C. balustinum*, but not salt stress, modified the pattern of flavonoids exuded by the bean roots. The capacity of flavonoids from root exudates to induce the expression of the *nod* genes and the production of lipochitooligosaccharides (LCOs) was investigated in two bean-nodulating rhizobia, *Rhizobium tropici* CIAT899 and *Rhizobium etli* ISP42. The *R. etli* ISP42 LCOs profile changed when it was induced by the exudates collected in the presence of the PGPR with and without salt. In the case of *R. tropici* CIAT899, changes in the LCOs profile were detected when the bacterium was induced with the different bean root exudates in comparison with the LCOs synthesized upon induction with apigenin.

1. Introduction

Plants produce a remarkably diverse array of about 100,000 low-molecular mass natural products also known as secondary metabolites (Dixon, 2001). These are organic compounds and inorganic ions that change the chemistry and biology of the rhizosphere and enhance adaptation to a particular environment (Crowley and Rengel, 1999). Plant exudates in the rhizosphere provide a rich source of energy and nutrients for bacteria, resulting in higher bacterial populations in this area (Gray and Smith, 2005). Rhizosphere microorganisms such as plant growth-promoting rhizobacteria (PGPR) appear to have the capacity to transform or modify the chemical composition of the rhizosphere (Pillai and Swarup, 2002).

PGPRs are selected as a result of processes of co-adaptation and co-evolution between plants and microorganisms that develop under the influence of the roots (Lucas-García et al., 2001). Plant growth-promoting capacity is related to different PGP physiological activities: (i) synthesis of growth-promoting substances, such as phytohormones (cytokinins, gibberellins, and auxins); (ii) enhancement of factors affecting mineral nutrition, such as phosphorous solubilization and biological nitrogen fixation; (iii) protection of plants against phytopathogens (Persello-Cartieaux et al., 2003; Somers et al., 2004). PGP bacteria can also protect plants against the effects of some environmental stresses such as heavy metals, salinity, and water stress (Mayak et al., 2004a,b). However, relatively few mechanisms have been unequivocally demonstrated to be an explanation for the increased resistance to environmental stresses of plants treated with PGPRs. Lucas-García et al. (2004) showed the effects of three PGPRs on nodulation and growth promotion in soybean plants. Thus, *Chryseobacterium balustinum* Aur9,

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a strain isolated from the rhizosphere of *Lupinus albus*, induced physiological changes that led to an enhancement of the biological nitrogen fixation. Nevertheless, plant growth-promoting bacteria found in association with plants grown under chronically stressful conditions, including salinity, may have adapted to these conditions, and could provide significant benefits to plants.

Several signaling molecules, including flavonoids, isoflavonoids and phenolic compounds secreted by the plant root are able to induce the expression of rhizobial *nod* genes. In response to these compounds rhizobia produce a series of host-specific signal molecules, lipochitooligosaccharides (LCOs), also known as Nod factors (Schlaman et al., 1998). As a consequence, the formation of root or stem nodules occurs in response to the presence of rhizobia. The incorporation of atmospheric N_2 into organic material resulting from this *Rhizobium*-legume symbiosis is estimated to account for one third of the total nitrogen needed for world agriculture. This unique intracellular association contributes significantly to agricultural yields (de Hoff and Hirsch, 2003). The establishment and activity of the symbiotic interaction are extremely sensitive to salt stress. Salt affects plant growth and the nodulation process mainly by decreasing the number of deformed root hairs, limiting the sites for bacterial infection (Zhu, 2001). Previous work carried out in our laboratory showed that salinity diminished the capacity of soybean root exudates to induce *nod* genes and to produce LCOs (Dardanelli et al., 2009). However, information about bean exudates produced under saline conditions and their effect on production of signal molecules is still scarce.

The impact of many chemical signals on the ecology of the rhizosphere is not as yet well understood. It is not clear, for example, how microorganisms modify the chemical signals and what the impact of changes are in the rhizosphere community or the abiotic stress on the flavonoids-mediated communication. A better understanding of the biology of root exudation should contribute to improvement of crop adaptation to stressful environments, such as saline lands, and to more sustainable and profitable farming.

The aim of this work was to study the impact of saline conditions (50 mM NaCl) on the flavonoids exudation patterns of *Phaseolus vulgaris* (common bean), in the presence or absence of the PGPR *C. balustinum* Aur9, to elucidate whether the presence of the bacterium affects root exudates. These exudates were studied using HPLC coupled to ESI-MS/MS (Rijke et al., 2006). We concluded that the presence of *C. balustinum* Aur9 only caused small changes in roots exudation. Furthermore, saline stress did not affect the exudation of flavonoids in *P. vulgaris* in the presence or absence of this PGPR strain. We also studied flavonoids-mediated *nod* gene induction and analyzed the LCOs produced by bean-nodulating rhizobia.

2. Material and methods

2.1. Bacterial strains, medium and culture conditions

Bacterial strains used in this work were *C. balustinum* Aur9 (Gutiérrez-Mañero et al., 2003), *Rhizobium tropici* CIAT899 (Martínez-Romero et al., 1985), and *Rhizobium etli* ISP42 (Rodríguez-Navarro et al., 2000). All rhizobial strains were grown in B^- medium (van Brussel et al., 1990), and *C. balustinum* was grown in B^- medium with glucose instead of mannitol as the carbon source and supplemented with casaminoacids. Cultures were grown with shaking at 150 rpm at 28 °C, and concentrations of viable bacteria (c.f.u. mL^{-1}) were obtained by plate counts on TY agar (Beringer, 1974). Bacterial growth was evaluated by measuring absorbance at 600 nm.

2.2. Growth of seedlings and preparation of bean root exudates

P. vulgaris Bush Blue Lake 274 (Fitó), seed surface disinfection, germination, and growth on nitrogen-free medium have been described elsewhere (Albareda et al., 2006). Twenty pregerminated seeds were aseptically transferred to a stainless-steel lattice placed in a glass cylinder containing 130 mL of a modified nitrogen-free Rigaud-Puppo solution 0.25× ($CaCl_2 2H_2O$ 25 mg L^{-1}, $MgSO_4 7H_2O$ 50 mg L^{-1}, KH_2PO_4 50 mg L^{-1} $NaHPO_4 2H_2O$ 18.75 mg L^{-1}, Gibson solution 1 mL L^{-1}, sequestrene 2.5 mg L^{-1}, pH 6.8) (Rigaud and Puppo, 1975). Plants were maintained in a growth chamber at 24 °C (14 h) and 20 °C (10 h) for 7 days. Bean root exudates (BRE) were collected by centrifugation at 6200 × *g* for 15 min, and the supernatants sterilized by membrane filtration (8 and 0.2 μm pore size cellulose nitrate filters). To check sterilization, 100 μL of BRE were inoculated in TY medium and incubated overnight at 28 °C. Sterile BRE were stored at 4 °C or −20 °C.

The hydroponics systems were inoculated, when required, with *C. balustinum* Aur9 at 10^6 c.f.u. mL^{-1}. Four different bean root exudates were obtained from five replicates: BRE uninoculated (BRE), BRE inoculated with *C. balustinum* (BREI), BRE uninoculated in the presence of 50 mM NaCl (BRES), and BRE inoculated with *C. balustinum* and in the presence of 50 mM NaCl (BREIS). The final concentration of *C. balustinum* in suspension in the hydroponics systems was 2.1×10^7 c.f.u. mL^{-1}. BRE acidified the nutrient solutions in the presence or absence of *C. balustinum* (from pH 6.8 to pH 6.4 and 4.9, respectively) and when the bean plants were exposed to salt stress (from pH 6.8 to pH 6.2 and 5.5, respectively). This experiment was performed three times.

2.3. Flavonoids sample preparation and analysis

One sixth of the lyophilized solid BRE, from five independent hydroponics cultures (300 mL) was resuspended in 50 mL of water and passed through a Resprep C18 solid phase extraction cartridge (Restek Corp., Bellefonte, USA). Flavonoids were released with 5 mL elution of 50%, 80%, and 100% methanol. These fractions were combined and freeze-dried. For HPLC analysis, samples were resuspended in 1 mL 50% methanol and 100 μL aliquots were injected into a HPLC-ESI system (Ríos et al., 2005). Sixteen standard flavonoids (supplied from Fluka or Sigma–Aldrich) were used: flavanones such as naringenin and hesperitin; flavonols such as kaempferol, fisetin, morin, and quercetin; isoflavones such as genistein and daidzein; flavones such as 7,4′-dihydroxyflavone, apigenin, chrysin, and luteolin; chalcones such as isoliquiritigenin; coumaronochromones such as lisetin; coumestans such as coumestrol, and coumarins such as umbelliferone. The flavonoid-glycosides genistin, daidzin and naringin were also used.

2.4. HPLC coupled to mass spectrometry

Chromatographic separation was performed using a Pelkin Elmer Series 200 HPLC system (Waltham, USA) coupled to a 2000 QTRAP hybrid triple-quadrupole-linear trap mass spectrometer (Applied Biosystem, Foster City, USA) equipped with an electrospray ion source.

HPLC analyses were performed on a 250 mm × 2.1 mm Tracer Spherisorb ODS2 C18 reversed-phase column with a particle size of 5 μm (Teknokroma, Barcelona, Spain). The flow rate was 0.3 mL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) 50:50 (v/v) acetonitrile:methanol. Both components contained 0.1% formic acid (v/v). The elution profile was: isocratic for 5 min with 5% B, linear for 15 min up to 55% B, linear for 25 min up to 100% B, and isocratic for 5 min (100% B).

Mass spectrometric detection was performed in the negative ion mode after electrospray ionization. For HPLC coupled to mass spectrometry analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ionspray voltage −4500 V, source temperature 300 °C, source gas 20 psi, declustering potential −70 V, and entrance potential −10 V. Collision-induced dissociation (CID) was performed with the following parameters: collision cell exit potential −15 V, and collision energy −35 V.

2.5. Determination of β-galactosidase activity

β-Galactosidase activity assays using the strains *R. tropici* CIAT899 and *R. etli* ISP42, both harboring plasmid pMP240 that carries a *nodA*::*lacZ* transcriptional fusion (de Maagd et al., 1988), were carried out as described by Zaat et al. (1987). β-Galactosidase activities were measured at least six times from samples obtained in duplicate.

2.6. Reverse phase thin layer chromatography (RP-TLC) analysis of lipochitooligosaccharides (LCOs)

Rhizobial strains were grown in B⁻ broth or in the different bean root exudates. Nodulation factors were labeled *in vivo* and analyzed by TLC using the procedure described by Spaink et al. (1992). For the radiolabeling of lipochitooligosaccharides, 1 μCi of glucosamine hydrochloride ^{14}C (specific activity 52 mCi mmol^{-1}, Amersham) was used. TLC plates (Silica gel 60 RP-18F254s, Merck) were exposed to Kodak X-Omat R films for 15 days and then developed (Spaink et al., 1992). Chromatography was performed three times from samples obtained in triplicate.

3. Results

3.1. Detection of flavonoids in bean root exudates

We aimed to elucidate whether or not abiotic stress and the presence of plant growth-promoting rhizobacteria could modify the production of flavonoids by common bean plants. Sixteen flavonoids were used as standards for the HPLC coupled to ESI-MS/MS analysis. The MS/MS spectra obtained allowed us to set up the Q1/Q3 ions for monitoring (data not shown). Additionally, the retention time was used to indicate the presence of glycosilated flavonoids, which are more water soluble than the free aglycone (Shaw et al., 2006). Thus, when standard glycosilated flavonoids were studied by HPLC coupled to ESI-MS/MS, earlier HPLC retention times (about 5 min) or their equivalent aglycones were obtained. This difference arises, probably, from the increased polarity due to the sugar moiety (Shaw et al., 2006). Nevertheless, the fragmentation patterns of both glycosilated and aglycones are identical, since the sugar moiety is lost during the first ionization (Q0) (Ríos et al., 2005). As a result, the MRM method allows the identification of any flavonoid glycoside by using its aglycon Q1/Q3 ions (Fig. 1).

Several flavonoids included in the standard set, such as genistein, coumestrol, daidzein, and luteolin were not detected, although their presence in bean root exudates have been previously described (Broughton et al., 2000). In contrast, three flavonoids were detected in these exudates for the first time: umbelliferone, 7′,4-dihydroxyflavone, and hesperetin. There were no differences in the pattern of flavonoids detected in the bean root exudates collected under salt stress when compared to the control conditions (Table 1). However, the presence of *C. balustinum* Aur9 influenced the quality of flavonoids produced by the common bean. Thus, quercetin and isoliquiritigenin were not detected in bean root exudates inoculated with *C. balustinum* (BREI). In addition, apigenin could be found in BREI but not in the other bean root exudates. This flavonoid was not detected when the exudates of *P. vulgaris* were obtained under saline conditions in the presence of *C. balustinum*. However, isoliquiritigenin could be detected again under these conditions (Table 1).

Table 1
Flavonoids detected in *Phaseolus vulgaris* cv. Bush Blue Lake (Fitó) exudates grown under control conditions, with 50 mM NaCl, in the presence of *Chryseobacterium balustinum* Aur9, and in the presence of *C. balustinum* Aur9 and 50 mM NaCl.

	Control	NaCl	Aur9	Aur9 + NaCl
Flavones				
Apigenin	–	–	+	–
7′,4-Dihydroxyflavone	+	+	+	+
Flavonols				
Quercetin	+	+	–	–
Flavanones				
Naringenin	+	+	+	+
Hesperetin	+	+	+	+
Chalcones				
Isoliquiritigenin	*	*	–	*
Coumarins				
Umbelliferone	+	+	+	+

The presence (+) or absence (–) of a flavonoid is indicated. Asterisks indicate that the flavonoid is glycosidated.

3.2. Growth of R. tropici CIAT899 and R. etli ISP42 in bean root exudates

Following the observation that there were changes in the pattern of flavonoids produced by *P. vulgaris* cv. BBL in the presence of *C. balustinum* (Table 1), we studied the impact of the exudates on *R. tropici* CIAT899 and *R. etli* ISP42 growth. Both bean-nodulating bacteria grew in the different bean root exudates as sole carbon sources, reaching an OD_{600} of about 0.2–0.3. This poor growth could be due to low carbon source concentrations in the bean root exudates. To optimize bacterial growth, BRE and BREI were mixed with B⁻ medium in a 1:1 proportion and bacterial growth was measured. The presence of salt in the root exudates did not affect bacterial growth (Fig. 2). After 72 h, both *R. tropici* CIAT899 and *R. etli* ISP42 populations were about 10^7 c.f.u. mL^{-1} in the different root exudates mixed with B⁻ medium and the biomass decreased approximately 80% when compared to the biomass reached in B⁻ medium (data not shown).

3.3. Capacity of the bean root exudates for inducing the expression of the nodulation genes and LCOs production

Since the flavonoids exudation pattern of common bean plants changed due to the presence of *C. balustinum* with and without salt stress, we investigated the capacity of the different root exudates to induce *nod* genes expression and the production of LCOs in two bean-nodulating rhizobia. Although both strains did not grow properly in the different bean root exudates, this growth was enough to support the production of LCOs. Results obtained are shown in Fig. 3. When *R. tropici* CIAT899 was induced with BRE collected under control conditions (Fig. 3A, lane 3), the spot of greater migration detected after induction with apigenin (Fig. 3A, lane 1), which has been assigned as corresponding to a hydrophilic family of structures containing sulphated compounds (Folch-Mallol et al., 1996), could not be detected. More significant changes were obtained when this rhizobial strain was induced with BRE collected in the presence of *C. balustinum* (Fig. 3A, lane 4). Thus, radioactive spots could not be observed, even though the *nod* gene expression level was similar to that observed in the absence of the PGPR (Fig. 3C, lanes 3 and 4). Under salt stress conditions, Nod factors produced

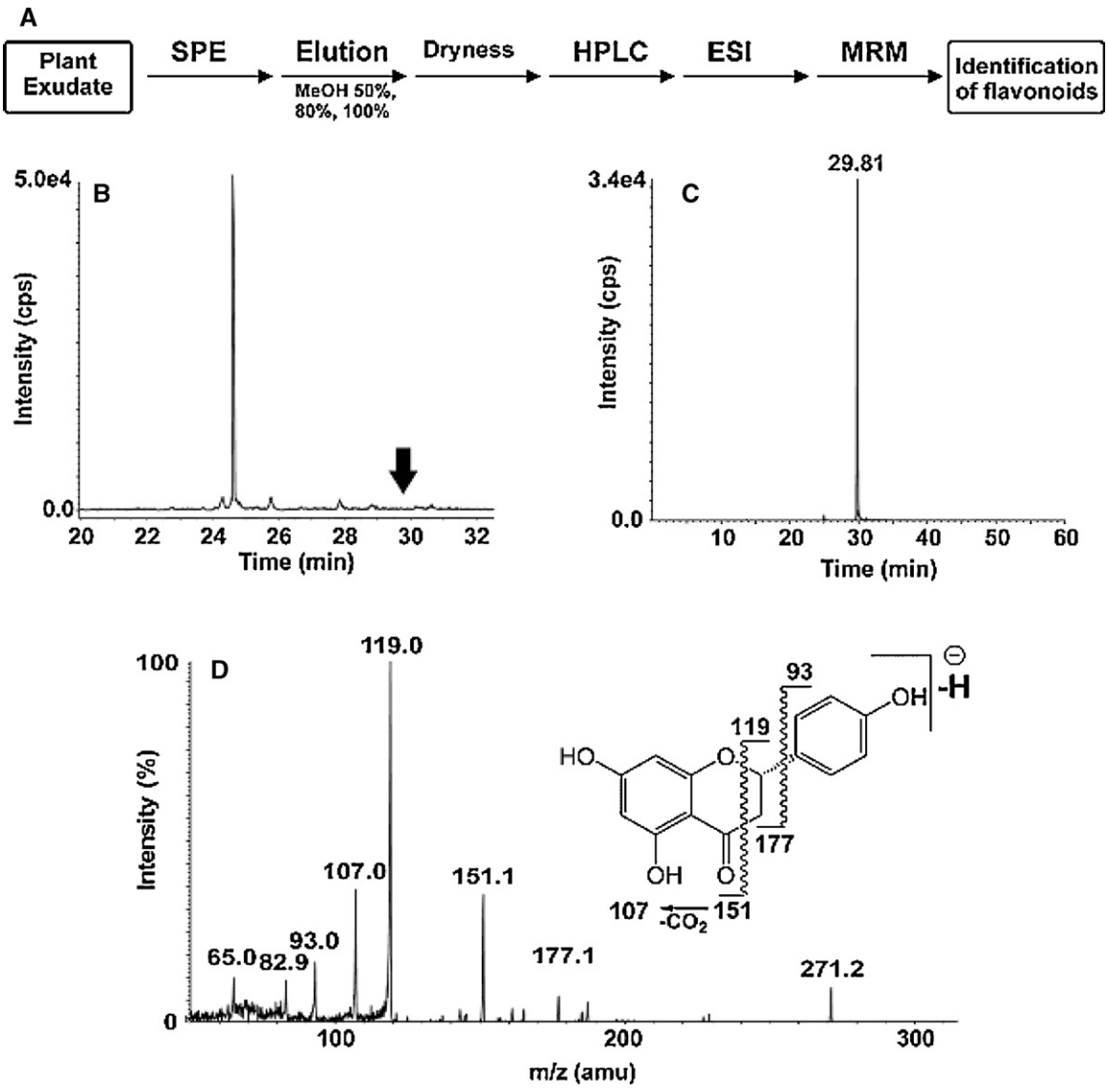

Fig. 1. Analysis the bean root exudates. (A) Scheme of the treatment applied to plant exudates; (B) HPLC chromatogram obtained when registering in Total Ion Monitoring mode; (C) HPLC chromatogram obtained when Multiple Reaction Monitoring is applied; the peak at 29.81 min (indicated in B) corresponds to naringenin; (D) Collision Induced Decomposition mass spectrum obtained from naringenin peak; product ions are assigned according to Fabre et al. (2001) and indicated in the structure.

by *R. tropici* CIAT899 in B^- medium and in BRE were identical to those produced without salt (Fig. 3A, lanes 1, 5 and 7). However, under salt stress conditions and in the presence of Aur9, the smaller band detected in BRE and BRES could also be detected (Fig. 3A, lane 8).

On the other hand, *R. etli* ISP42 showed significant variations in its LCO profile depending on the experimental conditions. Under control conditions (Fig. 3B, lanes 1–4) *R. etli* ISP42 produced LCOs when it was induced with BRE or naringenin (Fig. 3B, lanes 1 and 3). However, when the rhizobial strain was induced with BRE collected in the presence of *C. balustinum* no spots were observed (Fig. 3B, lane 4). In fact, this exudate weakly induced the expression of the *nod* genes (Fig. 3D, lane 4). In marked contrast to *R. tropici* CIAT899, the chromatographic profile of LCOs produced after induction with bean root exudates was similar to that observed after induction with commercial flavonoids (Fig. 3B, lanes 1 and 3). When exudates were obtained under salt stress conditions (BRES) only the central spot could be detected (Fig. 3B, lane 7). In the other experimental conditions assayed, no differences were observed with respect to the control conditions (Fig. 3B).

4. Discussion

Several techniques for studying root exudation are not routinely used due to numerous technical problems in collecting and analyzing root exudates (Crowley and Rengel, 1999). Moreover, quantitative collection of root exudates from soil-grown plants has little chance of ever being achieved. As a consequence, plants are often grown in nutrient solutions and the root exudates are collected from them (Rengel, 2002). For years, liquid chromatography coupled to UV detection has been the preferred method for the analysis of flavonoids. However, although UV spectra distinguishes among sub-classes, they are unable to identify individual flavonoids in most cases, requiring their isolation and further study off-line by other structural determination methods such as NMR or mass spectrometry. Moreover, the use of electrospray (ESI) as ion source largely increases the sensitivity, whereas application of tandem mass spectrometry (MS/MS) enables specific compounds to be detected in complex mixtures due to their specific and characteristic fragmentation patterns. This technique has been successfully applied to the study of other components of root exudates such

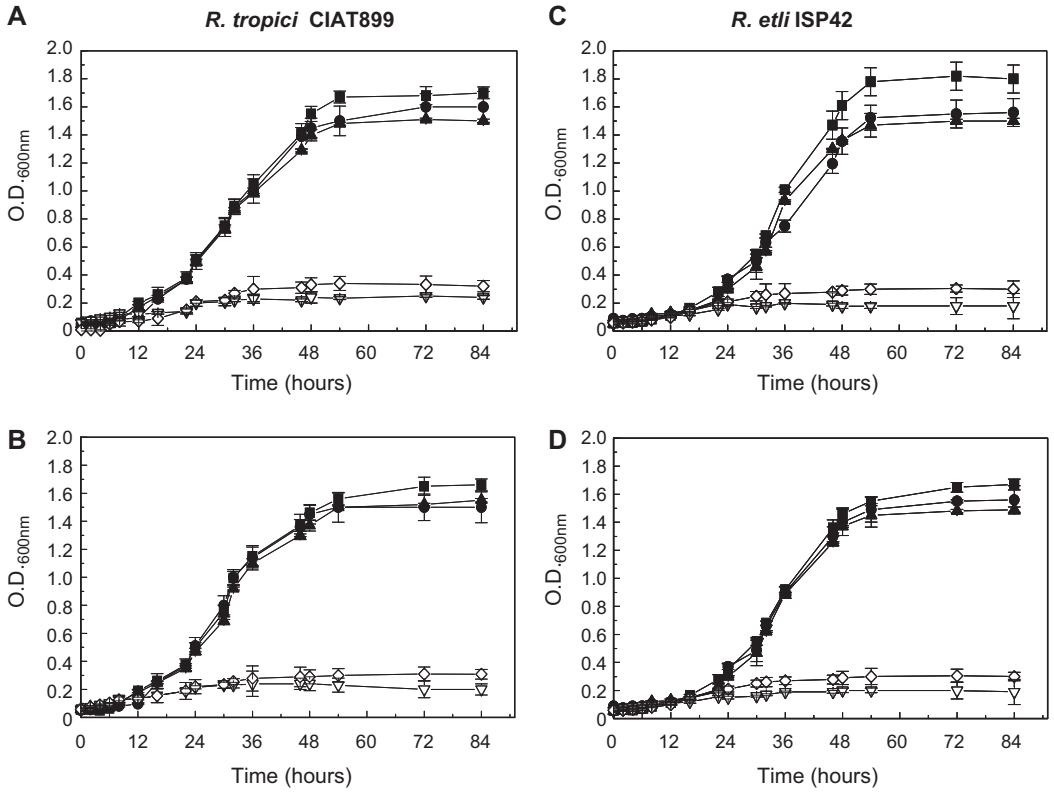

Fig. 2. Growth curves of *Rhizobium tropici* CIAT899 (A and B) and *Rhizobium etli* ISP42 (C and D) in different bean root exudates in the absence (A and C) or presence of 50 mM NaCl (B and D). Mineral medium B^- (black square), BRE (triangle), BREI (diamond), B^-: BRE (1:1) (black circle), B^-: BREI (black triangle). Growth curves show the mean $OD_{600\,nm}$ (±standard deviation of the mean) for three replicates.

as *N*-acyl homoserine lactones (Scott et al., 2006) or strigolactones (Awad et al., 2006), as well as to the study of flavonoids extracted from plant tissues (Kachlicki et al., 2005; Li et al., 2006), animal sources (Fang et al., 2006), and also to the study of flavonoids in soybean root exudates (Dardanelli et al., 2009).

The aim of this study was to study *P. vulgaris* cv. BBL (common bean) roots exudation of *nod* gene inducing compounds in response to inoculation with the PGPR *C. balustinum* Aur9 under either control or salt stress conditions, since exudate composition is known to change under diverse environmental stresses (Jones et al., 2004). Diverse flavonoids from root exudates of different common bean cultivars have been described as *nod* gene inducing factors (Hungría et al., 1991a,b; Dakora et al., 1993; Burdman et al., 1996; Bolaños-Vásquez and Werner, 1997). The sensitivity of the analytical method used in this work allowed the identification of three flavonoids that were not previously detected in common bean root exudates: umbelliferone, 7′,4-dihydroxyflavone, and hesperetin.

Experiments carried out in hydroponics systems have shown that inoculation with *Azospirillum* or *Pseudomonas fluorescens* increases secretion of *nod* genes inducing flavonoids by bean seedlings and chickpeas, respectively (Burdman et al., 1996; Andrade et al., 1998). Some differences in the flavonoids detected in BRE collected in the presence of *C. balustinum* versus BRE collected in the absence of this bacterium were observed, regardless of the presence of salt (Table 1). Flavonoids, besides acting as key signals for the initiation of nodule formation in the *Rhizobium*-legume symbiosis (Broughton et al., 2000; Cooper, 2007), can also represent a rich carbon source for those rhizosphere microorganisms that possess the appropriate catabolic enzymes. In this sense, the presence of microorganisms is known to influence the quality and quantity of flavonoids present in the rhizosphere, both through modification of root exudation patterns and microbial catabolism of exudates (Shaw et al., 2006). Thus, in the presence of *C. balustinum* and in the absence of salt a new flavonoid, apigenin, was identified, while others such as quercetin and isoliquiritigenin were not detected. These results indicate that the bacterium could modulate the production of flavonoids in *P. vulgaris* cv. BBL plants. We cannot conclude that changes in flavonoids exudation in the presence of a microorganism using a hydroponics system could also happen in the rhizosphere under natural conditions. In this work we show that the plant responds to a determined plant–bacteria interaction changing the pattern of exuded flavonoids. It is probable that the plant response to the presence of rhizosphere bacteria could be similar to that observed in our laboratory conditions. Kumar et al. (2006) proposed quercetin as a bacterial growth stimulator in the rhizosphere. Therefore, it might be possible that this flavonoid was consumed by *C. balustinum* during the exudation process. Under similar conditions, Dakora and Phillips (1996) and Aoki et al. (2000) reported the accumulation of isoflavonoids in infected plant tissues, instead of being secreted to the rhizosphere. Therefore, the variety and concentration of flavonoids could diminish, as we have also observed in the present work. In addition, the exudates collection method could modify the secretion or identification of flavonoids as well (Kamilova et al., 2006).

Once mass spectrometry studies confirmed changes in the flavonoids exudation pattern, β-galactosidase activity assays and LCOs analyses were performed with two bean-nodulating bacteria to identify possible modifications of the *nod* gene induction capacity of these exudates. Exudates have been used to study whether the effects caused by flavonoids exuded were synergistic, antagonistic or both (Hungría et al., 1992). Indeed, it is the mixture of inducing and inhibiting compounds present in the root exudate that is thought to control the overall level of *nod* gene induction (Zuanazzi et al., 1998). Although bean roots exuded the same flavonoids in the presence or absence of salt, a slight decrease in the induction capacity of the exudates collected under salt stress

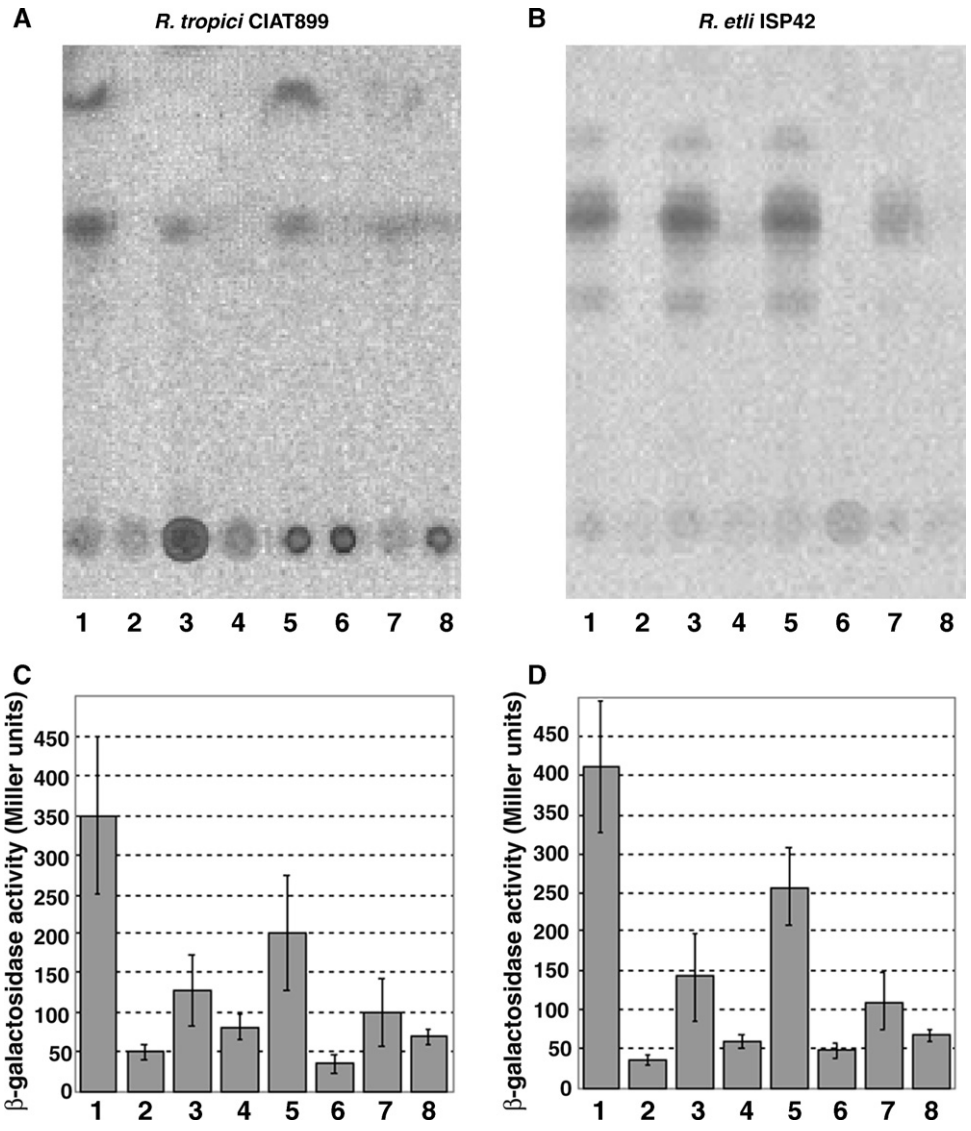

Fig. 3. Effect of *Chryseobacterium balustinum* Aur9 on bean root exudates produced under control (BRE) and salt conditions (50 mM NaCl, BRES). A and B, RP-TLC analysis of *Rhizobium tropici* CIAT899 and *Rhizobium etli* ISP42 LCOs labeled with ^{14}C *N*-acetylglucosamine. Panels C and D show the mean (±standard deviation of the mean) of the β-galactosidase activity of *R. tropici* CIAT899 (pMP240) and *R. etli* ISP42 (pMP240), respectively. Lanes 1 and 2, B^- medium; lane 3, BRE; lane 4, BREI; lanes 5 and 6, B^- medium 50 mM NaCl; lane 7, BRES; lane 8, BREIS. Lanes 1 and 5, LCOs obtained in the presence of the appropriate inducer at a final concentration of 1 μM (apigenin for *R. tropici* CIAT899 and naringenin for *R. etli* ISP42).

conditions was observed when compared to those collected under control conditions (Fig. 3B and D). These results are in agreement with those obtained by El-Hamdaoui et al. (2003), who reported a decrease in the induction capacity of chickpea exudates collected in the presence of 75 mM NaCl. Similar assays were performed with exudates collected in the presence of *C. balustinum*. Fig. 3 shows a decrease in the induction capacity of the exudates collected in the presence of the PGPR when compared to those collected in its absence, both under control or salt stress conditions. This decrease was more remarkable in the case of *R. etli* ISP42 (Fig. 3D, lanes 3, 4, 7, and 8). The cultivation period of 7 days allowed the production of metabolites at a sufficient concentration for the structural elucidation of flavonoids. However, it is possible that the original pattern of flavonoids in the cultivation system was the same regardless of the presence of the PGPR, but varied due to the metabolism of these flavonoids by the bacteria. This conclusion is not only supported by the mass spectrometry analysis, but also by several reports that confirm the biodegradation of legume flavonoids by rhizobacteria leading to secondary metabolites with different induction capacities. Rao and Cooper (1995) highlighted the potential consequences of microbial transformation of pre-existing flavonoid pools, namely the production of *de novo* flavonoids which could be either *nod* gene inducers or repressors.

With respect to the production of Nod factors, *R. etli* ISP42 profile changed both in intensity and in the number of radioactive spots when it was induced by the exudates collected in the presence of the PGPR with and without salt. In the case of *R. tropici* CIAT899, changes in the LCOs profile were observed when the bacterium was induced with bean root exudates. Moreover, these changes were more remarkable when the bacterium was induced with BREI (Fig. 3A and B).

We initially believed that the reporter *nodA*::*lacZ* fusion could be used to quantify the induction capacity of flavonoids produced by bean plants under different environmental conditions (both biotic and abiotic stress). However, further assessment revealed that complex and multiple mechanisms operate in regulating *nod* gene expression (Loh and Stacey, 2003) and the interaction between flavonoids may inhibit induction (Peters and Long, 1988) whereas non-flavonoid compounds may also act as inducers (Phillips et al., 1994). Consequently, no linear relation between exudate

concentration and β-galactosidase activity could be observed, since Novák et al. (2004) have already reported for chickpea plants. Non-specific binding or degradation of inducers by bacteria (Cooper and Rao, 1995) is another possible source of non-linearity. This notion is supported by a lower β-galactosidase signal at higher densities of the bacterial suspension.

5. Conclusions

The presence of *C. balustinum* in the *P. vulgaris* rhizosphere affects the pattern of flavonoids exuded by this plant. This effect may be due to the induction of new flavonoids biosynthesis pathways, to a PGP effect of C. *balustinum* that increases root surface, or to microbial catabolism of the flavonoids exuded by *P. vulgaris*. Changes in the pattern of flavonoids observed are not related to the presence or absence of salt stress.

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